Molecular markers of sperm quality

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Light microscopic semen evaluation provides useful information about a given sperm sample, but due to its subjective nature has limited prognostic value for the reproductive performance of males or the outcome of assisted fertilization. Cryptic sperm abnormalities (occurring at the molecular level) are not easily detectable by light microscopy, but can be revealed by an array of biomarkers. The latter include fluorescent markers of acrosomal status, fluorochromes detecting altered sperm chromatin or DNA integrity, vital dyes revealing sperm mitochondrial activity, probes detecting apoptotic events, and antibodies detecting proteins that are either up- or down-regulated in defective spermatozoa. Many of the above biomarkers are best tested by flow cytometry, permitting rapid, automated, high throughput, objective measurement of the relative abundance of these biomarkers in semen. This review summarizes a strategy for the identification of novel male fertility/sperm quality biomarkers based on proteomic, biochemical and immunocytochemical analyses of defective spermatozoa. This approach identifies proteins or ligands uniquely associated with defective spermatozoa, regardless of whether they carry gross morphological defects or subtle, but critical hidden defects (e.g. DNA strand breaks) not detected with conventional, light microscopic analysis. Such markers, including ubiquitin, sperm thioredoxin SPTRX3/TXNDC8, 15LOX, and Lewis^y-terminated N-glycans, are associated with poor semen quality and reduced fertility, warranting a designation of "negative" markers of fertility. The significance of sperm cytoplasmic droplet, a structure that accumulates several of the discussed biomarker proteins, is also discussed with regard to sperm quality and fertility.

Introduction: Novel approaches to semen analysis

Light microscopic evaluation is a cornerstone of semen analysis in both farm animal and human andrology, providing useful information about a sperm sample. However, conventional sperm tests such as motility and morphology assessments rely on subjective evaluation of sperm traits and have limited prognostic value for the reproductive performance of males or for the outcome of assisted fertilization. Consequently, it is difficult to predict the reproductive performance of bulls or boars used for artificial insemination. Similarly, human male infertility could be misdiagnosed as idiopathic, as certain types of sperm abnormalities occur at the molecular level,

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sometimes in the absence of morphological manifestation detectable by light microscopy. To address this problem, structural and molecular anomalies of defective human spermatozoa can be revealed by an array of biomarkers. These latter include fluorescent markers of acrosomal status (e.g. lectins PNA and PSA), fluorochromes detecting altered sperm chromatin or DNA integrity (e.g. acridine orange/SCSA or TUNEL assays), vital dyes revealing sperm mitochondrial activity (JC-1, MitoTracker), probes detecting apoptotic events (FAS ligand, caspase substrates), and antibodies detecting proteins that are either up- or down-regulated in defective spermatozoa. Many of the above biomarkers are best tested by flow cytometry (Fig. 1 A), which in contrast to light microscopic analysis, permits a rapid, automated, and objective measurement of their relative abundance in thousands of cells per sample [reviewed by (Garner, 1997; Gillan et al., 2005)]. Traditional microscopic sperm evaluation allows subcellular localization of specific biomarkers. However, relative intensity of labeling is typically estimated on a small number of cells (e.g. 100-200 per sample) by light microcopy. Collectively, flow cytometry and epifluorescence microscopy, combined with biomarkers of sperm quality/fertility, are quickly becoming useful techniques in andrology. These efforts are now elevated by the introduction of innovative flow cytometric approaches and novel instrumentation. For example, the ImageStream instrument combines the throughput of a flow cytometer with the rapid image acquisition capabilities of a high end CCD camera (Buckman et al., 2009), and the affordable, easy-to-use, but highly accurate capillary-based flow cytometers such as Guava PCA and Guava EasyCyte Plus (Polina et al., 2008) have the potential to be widely used in andrology . However, additional biomarkers of sperm quality are sought, suitable for flow cytometry, and highly correlated with field fertility of male farm animals.

Negative marker approach

We built our strategy for the identification of novel male fertility/sperm quality biomarkers on proteomic, biochemical, and immunocytochemical analyses of defective spermatozoa. We look for proteins or ligands that are uniquely associated with the defective spermatozoa carrying gross morphological defects, or subtle, but critical hidden defects (e.g. DNA strand breaks), not detectable in conventional, light-microscopic analysis. Such markers are associated with poor semen quality and reduced fertility, warranting a designation of "negative" markers of fertility.

To date, we have identified the following potential male fertility markers:

- Ubiquitin (Fig. 1 A, B) is associated with the surface of defective spermatozoa of humans (Sutovsky et al., 2001b), bulls (Sutovsky et al., 2002), boars (Lovercamp et al., 2007a; Sutovsky et al., 2003), stallions (Sutovsky et al., 2003), and rats (Tengowski et al., 2007).
- The 15-lipoxygenase (15LOX) is enriched along with various ubiquitin-interacting components in the cytoplasmic droplets of boar spermatozoa (Fischer et al., 2005) and can be used as a negative marker of boar semen quality (Lovercamp et al., 2007a). Male mice lacking the *15Lox* gene are subfertile, with a significantly increased post-testicular retention of total (i.e. combined proximal and distal) cytoplasmic droplets (Moore et al., 2010).
- We reported that a testis, male germ-line specific thioredoxin SPTRX3/TXNDC8 is associated with superfluous cytoplasm of defective human spermatozoa (Buckman et al., 2009; Jimenez et al., 2004), although this does not seem to be the case for farm animal species.
- In contrast to our negative marker approach, we found that platelet activating factor-receptor (PAFr), present on the normal sperm surface, is under-expressed on the surface of defective bull spermatozoa, although its presence on the surface of leukocytes contaminating some

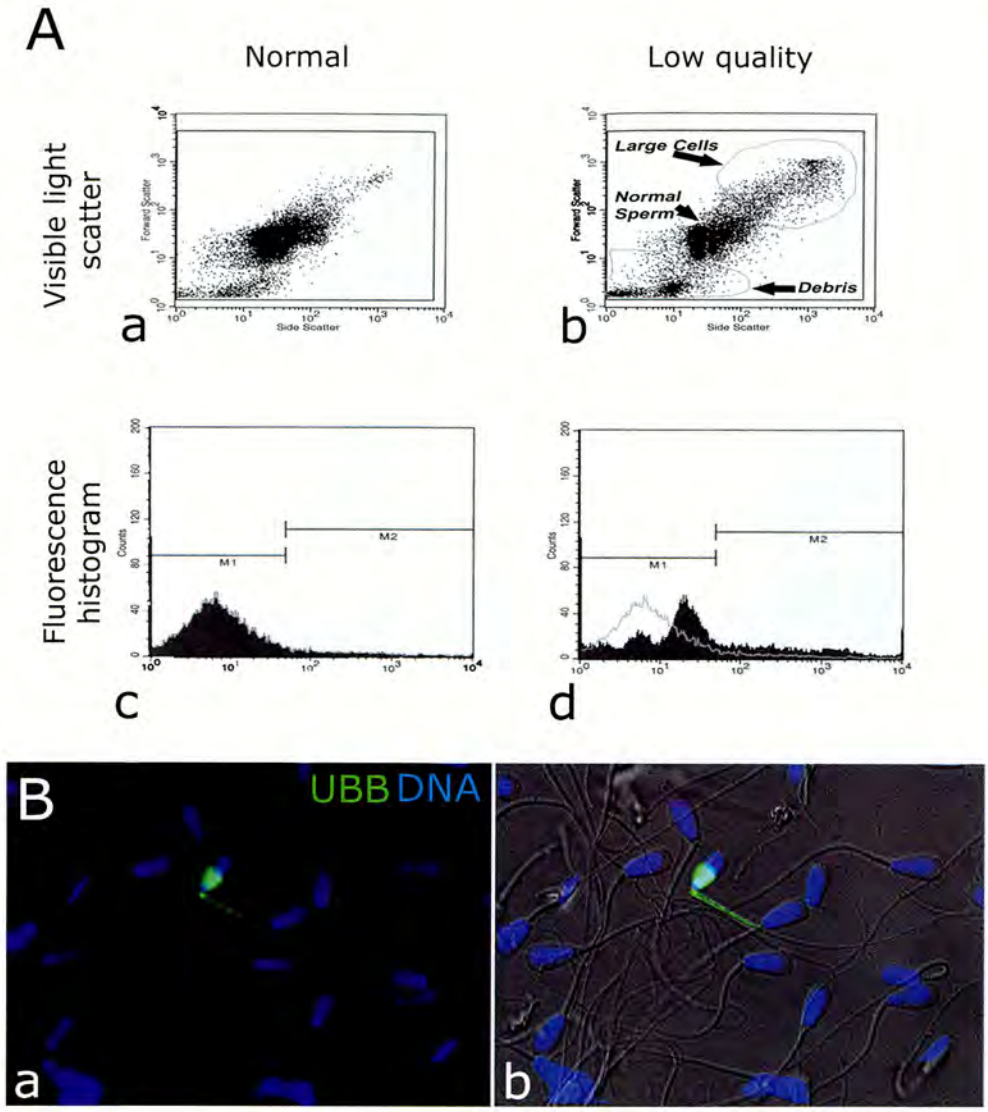

Fig. 1. (**A**) Flow cytometric analysis of bull sperm ubiquitin. Normal sperm sample from a fertile bull with median of ubiquitn-induced relative fluorescence (Ubiquitin Median value; no units) of 6.7 is shown on the left (panels *a & c);* right column, panels *b & d,* is a sample from a bull with a high percentage of morphologically abnormal spermatozoa, reflected by Ubiquitin Median value of 20.5. Panels *a* and *b* combine forward and side scatter diagrams of 10,000 cells/events per sample passing through visible light during flow cytometry. Each dot represents one cell/event. The desirable, presumably morphologically normal spermatozoa ("normal sperm" in panel *b)* congregate in the center. Cellular debris is concentrated in the lower left corner. Abnormally large cells are projected in the upper right corner. Panels *c & d,* corresponding to scatter diagrams *a & b,* respectively, are flow cytometric histograms of ubiquitin, reflective of the fluorescence induced by the binding of fluorochrome-conjugated anti-ubiquitin antibodies to the surface of defective spermatozoa. Cells within markers M1 are presumed normal spermatozoa; cells within markers M2 are those tagged with ubiquitin. Note the shift of the histogram peak in panel d, reflective of an increased median value of ubiquitin-induced fluorescence (ubiquitin median) in this sample. (**B**) Immunofluorescence of ubiquitin (green) on the surface of a defective bull spermatozoon surrounded by normal spermatozoa. Sperm nuclear DNA was counterstained with DAPI (blue). In panel *b,* fluorescence is superimposed with differential interference contrast image of the same cells.

of the bull semen samples could cause an artefactual increase the reading of this molecule in samples of poor quality (Sutovsky et al., 2007).

- Arylsufatase A (ASA), another sperm surface protein associated with normal fertility (Wu et al., 2007), is ubiquitinated and thus diminished on the surface of defective bull spermatozoa (unpublished data).
- Various glycan-specific lectins and antibodies are being tested for their ability to recognize altered sperm surface glycosylation in defective spermatozoa. In particular, *Lens cullinaris* agglutinin (LCA) recognizes the surface of ubiquitinated, defective bull spermatozoa (Baska and Sutovsky, 2005); antibodies recognizing the Lewis^y terminated N-glycans bind predominantly to defective human spermatozoa (Pang et al., 2007).
- Acrosome-bining lectins PNA (peanut/ *Arachis hypogaea*-agglutinin) and PSA (*Pisum sativum* agglutinin) recognize glycans present on the outer acrosomal membrane of the sperm acrosome and in the acrosomal matrix, respectively. Consequently, such lectins have been used extensively to determine acrosomal status (intact, capacitated, exocytosed, damaged) in farm animal spermatozoa [e.g. (Cross and Watson, 1994)].

Ubiquitin as a biomarker of male semen quality and fertility

The ubiquitin-proteasome pathway (UPP) regulates many steps of reproductive processes, including, but not limited to, spermatogenesis, epididymal sperm maturation, fertilization, and preimplantation embryo development. Ubiquitin is a small chaperone protein that binds covalently to internal Lys-residues of proteins destined for proteolytic degradation by the 26S proteaome, a multi-subunit proteolytic holoenzyme. Proteins are targeted for ubiquitination due to misfolding, altered glycosylation, oxidation, disulfide-bond reduction, or developmental/cellular programming [reviewed by (Glickman and Ciechanover, 2002)]. Although it is sometimes perceived as a housekeeping pathway, the UPP thus serves a variety of precisely regulated signaling events during the cell cycle, transcriptional control, membrane receptor turnover, and is involved in a variety of cellular pathologies, including Alzheimer's disease, liver cirrhosis, and HIV infection [reviewed by (Hershko and Ciechanover, 1998)]. Ultimately, the tagging of substrate proteins with a multi-ubiquitin chain of four or more ubiquitin molecules predestines such proteins for proteolytic degradation by the 26S proteasome. The latter is a multi-subunit protease complex of ~2 MDa, composed of the 19S regulatory complex (17 subunits; role: ubiquitinated substrate recognition, deubiquitination and priming) and 20S core (14 subunits; role: substrate degradation). Some proteasome-substrate interactions and interactions between 19S subunits are mediated by the 19S ATP-ase subunits which require ATP. Other steps of substrate recognition and priming in the 19S complex (maintenance of non-ATPase 19S subunits), as well as the actual proteolysis in the 20S core, are not ATP-dependent [reviewed by (Voges et al., 1999)].

Consensus is mounting that the proteolysis of ubiquitinated sperm and oocyte proteins by the 26S proteasome is necessary for the success of mammalian fertilization, including acrosomal exocytosis (AE) and sperm-zona pellucida (ZP) penetration (Sakai et al., 2004; Zimmerman and Sutovsky, 2009). The participation of UPP is also required for elimination of paternal mitochondria after fertilization, an observation explaining the basic dogma of developmental and evolutionary biology, namely that of strictly maternal inheritance of human/mammalian mitochondrial DNA (Sutovsky et al., 1999). The validation of ubiquitin as a biomarker of sperm quality was inspired by observations that mammalian spermatozoa with a visible or hidden defect acquire a ubiquitin tag during epididymal sperm maturation (Baska et al., 2008; Sutovsky et al., 2001a). The UPP operates in the mammalian epididymal fluid by virtue of a

peculiar apocrine secretion mechanism by which all necessary enzymatic components of UPP are introduced in the epididymal fluid and are in contact with maturing spermatozoa (Baska et al., 2008). Secretion of ubiquitin and other proteins in epididymal fluid occurs through apical blebs (Hermo and Jacks, 2002), large membrane enclosed vesicles of epididymal cell cytoplasm in which both secretory and non-secretory proteins appear to be concentrated. Besides ubiquitin, the ubiquitin activating, ubiquitin-conjugating and deubiquitinating enzymes, as well as various subunits of the ubiquitin dependent protease, the 26S proteasome, have been detected in epididymal fluid (Baska et al., 2008; Jones, 2004). Proteomic analysis also identified ubiquitin in epididymosomes, small epididymal cell-derived vesicles implicated in transferring epididymal proteins to the sperm plasma membrane (Thimon et al., 2008). The concept of ubiquitination and proteasomal degradation occurring on the cell surface or in extracellular space is now being recognized as a non-traditional, extracellular function of UPP, extending beyond the reproductive system (Sakai et al., 2004; Sixt and Dahlmann, 2008).

What makes defective spermatozoa recognizable to UPP? In general, it is protein damage by misfolding/unfolding, oxidation, disulfide bond-reduction, deglycosylation, or sugar trimming (Glickman and Ciechanover, 2002). A classical example is the ubiquitination and degradation of misfolded amyloid proteins in the brain cells, which, when blocked, results in the formation of amyloid plaques and accumulation of undegraded proteins characteristic of Alzheimer's and Huntington's disease. Interestingly, amyloid-like protein aggregates have been recently found in epididymal fluid and proposed to be a part of the epididymal extracellular protein quality control (Cornwall et al., 2007). Regarding altered glycosylation, we observed that defective, ubiquitin tagged bull spermatozoa acquire the ability to bind lectin LCA [(Baska and Sutovsky, 2005) and unpublished data]. Lectin LCA has high affinity for sugar-trimmed mannose-rich glycoproteins known to be ubiquitinated during endoplasmatic-reticulum associated protein quality control [ERAD; reviewed by (Spiro, 2004)]. Perhaps sperm surface glycoproteins could be trimmed in defective epididymal spermatozoa by alpha-mannosidase, one of the abundant glycosidases in epididymal fluid. Alternatively, glycosylases from epididymal fluid could recognize the altered sperm surface and attach immunoprotective glycans to the sperm surface, as observed in defective human spermatozoa (Pang et al., 2007).

Studies of sperm ubiquitination in farm animal species

Flow cytometry has been used as an objective, automated, statistically robust measurement of the relative levels of the above sperm biomarkers in farm animal and human semen. The following observations were made from farm animal field studies:

- Ubiquitin correlates positively with bull sperm DNA fragmentation (Sutovsky et al., 2002).
- Ubiquitin levels change seasonally in stallions, mirroring the seasonality of stallion semen output and quality (Sutovsky et al., 2003).
- Ubiquitin correlates negatively with conventional semen analysis end points (sperm count, motility, percentage of normal morphology), but positively with relative levels of sperm surface associate platelet activating factor-receptor (PAFr) protein in yearling bulls (Sutovsky et al., 2007).
- Semen content of 15LOX measured by flow cytometry correlates negatively with litter size in pigs bred by artificial insemination, whereas ubiquitin has negative correlations with both litter size and farrowing rates. Both 15LOX and ubiquitin correlate positively with the percentage of spermatozoa with a cytoplasmic droplet (CD) in boar semen (Lovercamp et al., 2007a).

Significance of sperm cytoplasmic droplet for farm animal fertility

Ubiquitin and other components of UPP accumulate in bull and boar sperm CD (Fischer et al., 2005; Sutovsky et al., 2001a) and can be used for automated measurement of the semen content of spermatozoa bearing a CD in boars (Lovercamp et al., 2007a). Sperm-borne CD is one of the most widespread and poorly understood sperm abnormalities of boars and bulls. The CD is a membrane-enclosed vesicle of cellular cytoplasm which remains attached to the sperm tail midpiece after completion of spermatogenesis. Mammalian CD are generally spherical and 2-3μm in diameter (Kaplan et al., 1984). During microscopic evaluation, CDs are observed either attached or unattached to the spermatozoa. When attached to the spermatozoon, the CD is associated with the sperm tail midpiece in one of two locations: at the proximal end of the midpiece, i.e. next to the sperm head (proximal CD) located at the distal end of the midpiece (distal CD), or at the junction of the midpiece and principal piece of the sperm tail. The distal CD can also be associated with the sperm tail abnormality known as the distal midpiece reflex, characterized by a 180° bend of the sperm tail around the distal CD at the midpiece/principal piece junction (DMR CD). Finally, the CD can also be detached from the sperm tail and free-floating in the ejaculate (unattached CD). Unattached CDs can be observed individually or as clusters; the latter are present in boar semen.

Formation of CD occurs during spermatogenesis when the haploid round-shaped sperm cell, a round spermatid, matures into an elongated spermatid and eventually into a fully differentiated spermatozoon. Formation of the CD begins when the residual cellular cytoplasm is removed from the elongated spermatid and is retained and destroyed by the Sertoli cell, the nurturing cells of the seminiferous epithelium (Barth and Oko, 1989). When the spermatid stalk, which connects the residual cellular cytoplasm to the presumptive CD is severed, a small amount of cytoplasm remains attached to the sperm midpiece. This small amount of cytoplasm is the CD. All newly formed sperm cells in the testis possess a proximal CD following spermatogenesis (Russell, 1984).

After spermatogenesis, spermatozoa pass from the testis into the epididymis; as spermatozoa pass through epididymis (for 7 to 14 days, depending on the species), they undergo maturation, and become capable of fertilization. During this passage, the CD migrates along the midpiece from the proximal to the distal position. It is not yet understood why, or by what mechanisms, movement of the CD on the sperm tail midpiece occurs during epididymal transit. Following migration to the distal position, the CD can be released in the cauda region of the epididymis in some species. In contrast to the bull and other mammals, the majority of boar spermatozoa in the cauda epididymis still possess a CD in the distal position (Harayama et al., 1996), and some spermatozoa possess a CD in the proximal position. During ejaculation or soon thereafter, CDs can be released from the boar spermatozoa and found free-floating within the seminal plasma (Harayama et al., 1996). Extensive research has analyzed the migration and disposal of the CD in various animal species during epididymal transit, concluding that CDs shed by epididymal spermatozoa are subsequently phagocytosed by epididymal epithelial cells (Axner et al., 2002; Hermo et al., 1988; Temple-Smith, 1984). It is not known whether the CD is formed on the spermatozoa to serve a specific purpose during sperm maturation or fertilization, or if it is simply a rudiment remaining on the sperm cell after spermatogenesis is completed.

Post-epididymal retention of CD has been associated with reduced fertility in both bulls and boars. Both proximal and distal CDs are thought to affect sperm quality/fertility, though individual studies may attribute more weight to one form of CD over the other. Possible factors causing the retention of the CD by ejaculated spermatozoa include suboptimal temperature of semen (Zou and Yang, 2000), environmental temperature of the male (heat stress)(Akbarsha et al., 2000; Huang et al., 2000), reprotoxic chemical exposure (Akbarsha et al., 2000), sexual

immaturity (Amann et al., 2000; Arteaga et al., 2001), altered/poor diet (Hassan et al., 2004), disease such as PRRSV in boars (Prieto et al., 1996), photoperiod changes (Sancho et al., 2004) and irregular collection frequency (Pruneda et al., 2005).

There is a negative correlation between sperm cytoplasmic droplet retention by yearling bulls and embryo cleavage following bovine in vitro fertilization (Amann et al., 2000). Spermatozoa from bulls with a high percentage of retained CDs had reduced ability to bind to oocyte zona pellucida, resulting in lower fertilization and cleavage rates in vitro (Thundathil et al., 2001). Sperm-bound CDs in extended boar semen have detrimental relationships with pregnancy rates and litter size (Waberski et al., 1994). Furthermore, these authors noted that CDs represent the most frequent morphological abnormality in boar sperm used for AI. In an *in vitro* study (Petrunkina et al., 2001), using sperm binding to explants of the pig oviductal epithelium of the oviductal sperm reservoir, there was a significant negative correlation of the percentage of sperm with attached CDs and sperm-explant binding. Furthermore, there was a negative correlation between sperm motility and the percentages of spermatozoa with attached CDs. A negative relationship was found between boar sperm CD-frequency, and farrowing rate and total number born in an artificial insemination trial (Lovercamp et al., 2007a; Lovercamp et al., 2007b). Boars with a farrowing rate below the average for the study had a higher number of sperm with attached CDs, and an increased content of sperm ubiquitin compared to the boars above the average farrowing rate.

Conclusions and perspectives

Collectively, the present review illustrates the utility of negative sperm quality markers in male fertility evaluation in large animals. The major advantages of biomarker approach over conventional semen analysis is its ability to accurately, and objectively, measure biomarker levels in a large number of cells per sample, and to uncover hidden sperm defects, not manifested in abnormal morphology. An added advantage of these high precision biomarkers is the ability to discern subtle differences in fertility within the pool of fertile sires, as opposed to extreme differences between fertile and completely infertile animals, revealed by conventional analysis. Of interest is the characterization of biomarker patterns/levels in subfertile animals with compensable vs. non-compensable sperm defects, and the ability to identify sires with superior tolerance to semen cryopreservation, and those that can be distributed in AI doses at higher dilution/lower sperm count per dose. The long-term goal of this work is to determine which markers coincide most closely with pregnancy/non-return rates in cattle, and with pregnancy rates and litter size in pigs. This will facilitate commercialization and dissemination of simple, but highly accurate and objective testing methods for the AI industry and farm animal producers. Further efforts will be focused on understanding how these biomarkers correlate with transient impairments of male infertility caused by heat stress, malnutrition, poisoning or contagious diseases, and permanent fertility disorders caused by testicular or epididymal disfunction, or injury.

Based on the presence of several biomakers on the surface of defective spermatozoa, magnetic nanoparticle-based methods are in development for depletion of defective spermatozoa from semen samples during semen processing for cryopreservation. At the same time, efforts are increasing to promote the use of novel flow cytometric instrumentation in farm animal and human andrology. Besides targeting sperm proteins/surface ligands as biomarkers, genome and transcriptome analyses will increasingly affect the field of andrology. The analysis of sperm-borne RNAs in humans already revealed that, perhaps in parallel to our observations of increased protein ubiquitination in defective spermatozoa, ubiquitin-proteasome pathway

gene products are deregulated in the sperm transcriptomes of infertile men (Platts et al., 2007). At the gene level, single nucleotide polymorphisms (SNP) have been found in infertile men within the sequences of the genes encoding for ubiquitin-specific protease USP26 (Stouffs et al., 2005) and ubiquitin activating enzyme UBE2B (Huang et al., 2008). With the help of new SNP chip-technology, the whole genome SNP screening (Van Tassell et al., 2008) is likely to reveal additional genes linked to spermatogenesis and sperm quality traits, in bulls and other species. Some of these newly identified marker genes could translate into biomarker-based assays of sperm quality at the protein level.

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